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A novel *p53* mutational hotspot in skin tumors from UV-irradiated *Xpc* mutant mice alters transactivation functions

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A mutation in codon 122 of the mouse *p53* gene resulting in a T to L amino acid substitution (T122→L) is frequently associated with skin cancer in UV-irradiated mice that are both homozygous mutant for the nucleotide excision repair (NER) gene *Xpc* (*Xpc*$^{-/-}$) and hemizygous mutant for the *p53* gene. We investigated the functional consequences of the mouse T122→L mutation when expressed either in mammalian cells or in the yeast *Saccharomyces cerevisiae*. Similar to a non-functional allele, high expression of the T122→L allele in *p53*$^{-/-}$ mouse embryo fibroblasts and human Saos-2 cells failed to suppress growth. However, the T122→L mutant *p53* showed wild-type transactivation levels with *Bax* and *MDM2* promoters when expressed in either cell type and retained transactivation of the *p21* and the *c-Fos* promoters in one cell line. Using a recently developed rheostatable *p53* induction system in yeast we assessed the T122→L transactivation capacity at low levels of protein expression using 12 different *p53* response elements (REs). Compared to wild-type p53 the T122→L protein manifested an unusual transactivation pattern comprising reduced and enhanced activity with specific REs. The high incidence of the T122→L mutant allele in the *Xpc*$^{-/-}$ background suggests that both genetic and epigenetic conditions may facilitate the emergence of particular functional *p53* mutations. Furthermore, the approach that we have taken also provides for the dissection of functions that may be retained in many *p53* tumor alleles.
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Introduction

The tumor suppressor gene *p53* plays an important role in maintaining genome integrity (Prives and Hall, 1999). Upon stabilization and activation, which are achieved mainly by post-translational modifications (Meek, 1999), p53 protein can act in the homotetrameric conformation as a sequence-specific transcription factor (McLure and Lee, 1998). Transactivation is the best characterized and probably most relevant function among the many biochemical activities ascribed to p53 protein (Ko and Prives, 1996). p53-response elements (REs) corresponding to a rather loose consensus sequence (two variably spaced palindromic decamers of 5′-RRRCWWGYYY each binding to a p53 dimer) (el-Deiry *et al*., 1992) have been identified in promoter and intronic regions of over 50 genes. The growing list of p53-regulated genes includes *p21*, *GADD45*, *p53–R2*, *FAS*, *Bax*, *PIG3*, *IGF–BP3*, *Killer/DR5*, *AIP1* and *MDM2* (Ashcroft and Vousden, 1999; el-Deiry, 1998; Oda *et al*., 2000; Tanaka *et al*., 2000). The products of these genes are involved in cell cycle control, induction of apoptosis, modulation of DNA repair, differentiation, senescence, and control of p53 stability/activity.

Recent studies have further validated the view that p53 is a tightly controlled cellular secondary sensor that can integrate many different signals and that regulates the expression of a large number of genes with variable kinetics and intensity (Vogelstein *et al*., 2000; Yu *et al*., 1999; Zhao *et al*., 2000). This key role in signal transduction predicts a strong selection for p53 inactivation in tumors and supports the notion that loss of p53 function disrupts basic cellular functions. An issue that has received relatively little attention is that partial inactivation of p53 activity may be sufficient for, or even favor, tumor progression, depending on the cell type and physiological state, and the precise function of the mutant *p53* allele in question.

The *p53* gene is frequently (>50%) mutated during tumorigenesis (Hainaut and Hollstein, 2000). About 80% of p53 tumor mutations are missense and result in single amino acid changes that are predicted to interfere with DNA binding and hence impair transactivation (Ko and Prives, 1996). Some mutations

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can be dominant-negative leading to partially inactive heterotetramers (Aurelio *et al*., 2000; Brachmann *et al*., 1996), a feature that may explain the unusual predominance of missense changes in the *p53* tumor mutation spectrum (Hernandez-Boussard *et al*., 1999).

Several recent studies support the view that certain *p53* mutant alleles can retain partial function. Some mutations are associated with partial transactivation and are capable of inducing G1 arrest, but not apoptosis (Ludwig *et al*., 1996; Munsch *et al*., 2000). Furthermore, a recent analysis of 77 different p53 mutant proteins from tumors revealed that >15% could still activate a yeast promoter containing a *p21* RE, but not the *Bax* or *PIG3* RE (Campomenosi *et al*., 2001), which presumably is important in tumor development. The observation that deletion of the *p21* gene does not result in increased spontaneous tumor development and can even decrease tumorigenesis (Pantoja and Serrano, 1999; Wang *et al*., 1997), suggests that *p53* mutant alleles that retain some transactivation capability might confer a selectable advantage over null alleles under appropriate conditions. For example, apoptosis can be prevented by expression of *p21* (Gorospe *et al*., 1997; Suzuki *et al*., 2000).

A few *p53* mutant alleles appear to have acquired functions that may provide a selective advantage to tumor cells, such as the up-regulation of growth promoting genes (e.g., *Myc*, *MDR*, *VEGF*) (Frazier *et al*., 1998; Kieser *et al*., 1994; Lin *et al*., 1995). Finally, *p53* mutant alleles that appear normal for transactivation, growth suppression and apoptosis when ectopically expressed at high levels in a tumor cell line, have been detected in breast and ovarian tumors associated with *BRCA1* defects (Smith *et al*., 1999). Hence, detailed functional analysis of multiple tumor *p53* alleles is expected to provide valuable information in predicting tumor state, including aggressiveness and responsiveness to therapy.

We recently reported that codon 122 of the mouse *p53* gene is a novel hotspot for mutation in UV radiation-induced skin cancers in xeroderma pigmentosum (XP) group C mice that are also hemizygous for *p53* ($Xpc^{-/-}$ $p53^{+/-}$) (Reis *et al*., 2000). Surprisingly, the hotspot does not involve adjacent pyrimidines, the major targets for UV damage, but instead occurs at an AC site. The resulting threonine to leucine amino acid substitution (T122→L) accounted for ~40% of all mutations in skin cancers from UV-irradiated $Xpc^{-/-}$ $p53^{+/-}$ mice, and was never detected in UV radiation-induced skin cancers in wild-type mice. Similarly, the T122→L mutation accounted for only 6% of *p53* mutations in $Xpc^{+/-}$ $p53^{+/-}$ or in $Xpc^{-/-}$ $p53^{+/+}$ mice and was also only rarely observed in $Xpa^{-/-}$ mice or mice defective in the *Csa* (Cokayne syndrome group A) gene required for transcription-coupled excision repair (D Nahari and EC Friedberg, unpublished observations). The origin of this mutational hotspot is not known, but is presumed to result from a non-dipyrimidine photoproduct. Its unique prevalence in skin cancers from $Xpc^{-/-}$ $p53^{+/-}$ mice suggests both that the *Xpc* gene product is specifically required for its repair, and that the T122→L mutation is recessive. However, the prevalence of this signature mutation uniquely in the $Xpc^{-/-}$ $p53^{+/-}$ genetic background may additionally or alternatively be determined by novel specific functional attributes of the mutant p53 protein encoded by the T122→L allele that promote its selection during carcinogenesis in the skin. Furthermore, such selection may be influenced by physiological attributes associated with the *Xpc* homozygous mutant state.

In order to explore the functional consequences of the T122→L mutation we have carried out ectopic expression of this mutant p53 protein in mouse embryo fibroblasts (MEFs) and in human tumor cells that are *p53* null. Additionally, we have exploited a recently developed p53 functional assay in yeast (Inga *et al*., 2001) that was adapted to precisely assess the transactivation potential of the T122→L allele with various p53 REs under conditions of variable expression. Using this sensitive assay, the T122→L allele exhibits a novel set of functional changes embracing both enhanced and reduced transactivation activity for 12 different p53 REs. We conclude that the precise pattern of T122→L p53-regulated gene expression, rather than a simple loss of p53 function, may be a critical determinant in tumor selection and progression in UV-irradiated mouse skin cells.

Results

The T122→L allele exhibits limited growth suppression in MEF and Saos-2 cells

In order to functionally characterize the murine T122→L allele in mammalian cells it was transferred into $p53^{-/-}$ MEF cells using a retroviral vector that generates high levels of p53 expression. Responses were compared with expressed wild-type p53 and an R270→C mutant allele. Mouse codon R270 and the equivalent human codon R273 are mutational hotspots in *p53* that are known to inactivate p53 function in many types of tumors. Expression of wild-type p53 protein reduced the relative colony forming ability to 12% (Figure 1a). As expected, the R270→C allele was much less effective at causing growth suppression. Expression of the T122→L allele had an intermediate effect (65% vs 42% survival, relative to vector control), suggesting some retention of p53 function.

We also examined the effect of high expression of the T122→L allele in the human osteosarcoma-derived Saos-2 cell line which is null for both the *Rb* and the *p53* tumor suppressor genes (Figure 1b). Once again, wild-type p53 reduced relative colony formation while the T122→L and the R270→C alleles had less effect (70% vs 88% survival, respectively, relative to vector). Hence, as relates to growth the T122→L mutant protein has minimal residual function in both cell lines in this assay.

The expression levels of p53 protein were examined in stable MEFs and Saos-2 transformant clones obtained in a limiting dilution experiment. As expected,

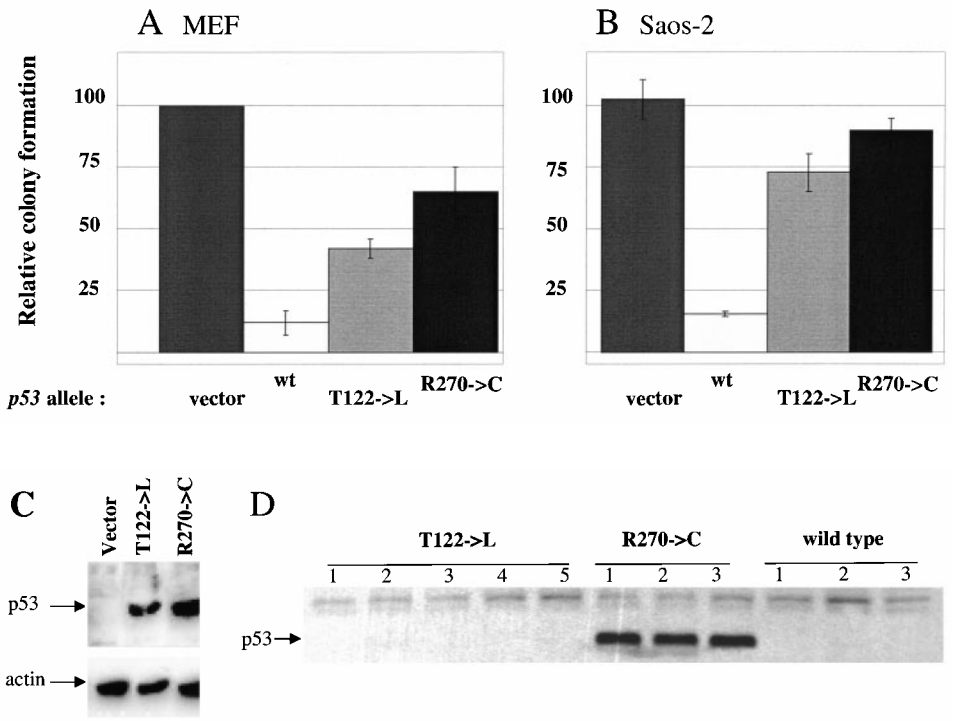


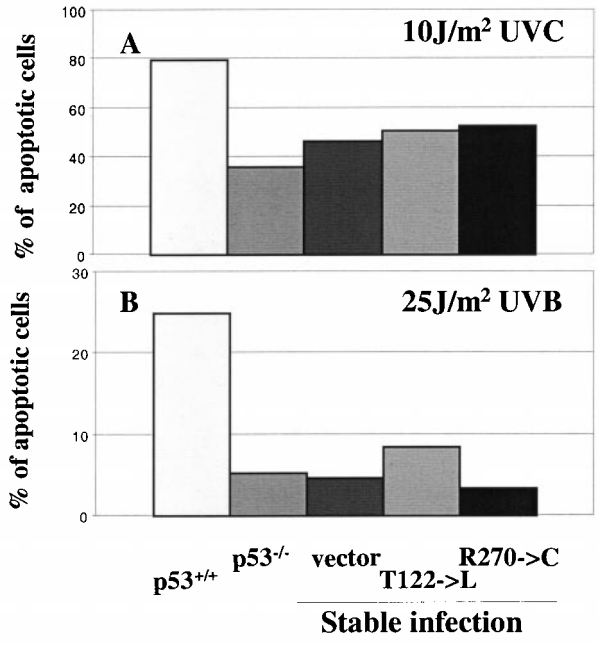
Figure 1 Growth suppression of $p53^{-/-}$ MEF and Saos-2 cells: (**a**) $p53^{-/-}$ MEF cells were infected by the retroviral vector pBabe–PURO expressing the indicated *p53* alleles under the LTR promoter. (**b**) Saos-2 cells were transfected by lipofectamine with 1 μg of pCI-based vectors (*CMV* promoter). Puromycin (**a**) and G418 (**b**) resistant colonies were selected and counted after 2 weeks. The relative mean number and the standard deviations for three independent experiments are presented. Expression of *p53* alleles in stable transfectants: puromycin resistant MEF clones (**c**) and G418 resistant Saos-2 clones (**d**) were isolated and p53 expression was determined by Western blot analyses (pAb1801 and DO-1). 50 μg of extract were loaded in each lane. Actin was used as control for MEFs extracts while the non-specific band served as control for the Saos-2

there was no detectable p53 in the isolates recovered from transfections with wild-type *p53*, while the R270→C transfectants expressed p53 protein at moderate levels (Figure 1c,d, and data not shown). T122→L protein expression was also retained in stable MEF clones (Figure 1c). In contrast, no p53 protein was detected in Saos-2 clones transfected with the T122→L vector (Figure 1d, five of the six clones examined are presented), suggesting that, similar to wild-type p53, the T122→L protein may not be tolerated in this cell line. Lower levels of expression from the LTR promoter compared to the CMV promoter (data not shown) may explain why stable transfectants expressing T122→L in the MEF cells could be established. However, the result is surprising since the presence of the T122→L vector had little effect on growth suppression in Saos-2 cells (Figure 1b).

The T122→L protein retains the ability to cause DNA damage-induced G1 arrest but not apoptosis

$p53^{+/+}$ and $p53^{-/-}$ MEF clones expressing either the R270→C or T122→L p53 alleles were irradiated with either 10 J/m^2 UVC or 25 J/m^2 UVB light and the number of apoptotic cells was measured 48 h later using FACS analysis. These doses resulted in different levels of both p53-dependent and -independent apoptosis (Figure 2a,b). The *p53* mutants were defective in apoptosis and exhibited levels of cell death comparable to *p53* null cells (i.e., p53-independent apoptosis).

Fibroblasts manifest a preference for p53-dependent cell cycle arrest over apoptosis when DNA damage is not extensive (MacCallum *et al*., 1996). Therefore, UVB radiation-induced cell cycle responses were examined in the non-apoptotic cell population 48 h after exposure to UV radiation of 25 J/m^2. Many of the MEF cells lacking *p53* were in the S phase (Figure 2c; black line in the top figure), indicating that the G1 arrest was not functional and that irradiation slowed progression through the S phase, leading to an accumulation of cells in this phase of the cell cycle. Mock-irradiated $p53^{-/-}$ cells were largely in G1 at 48 h, typical for cycling cells of this MEF line (solid peaks). As expected, irradiated normal MEFs ($p53^{+/+}$) that express the wild-type *p53* gene arrested in G1. T122→L-expressing cells similarly retained a large G1 population, consistent with retention of p53 function (Figure 2c). In contrast, cells expressing the R270→C allele exhibited a UV-radiation response similar to $p53^{-/-}$ MEFs (data not shown). Collectively, these results suggest that unlike the R270→C allele, the T122→L allele does not result in a typical loss of function phenotype.



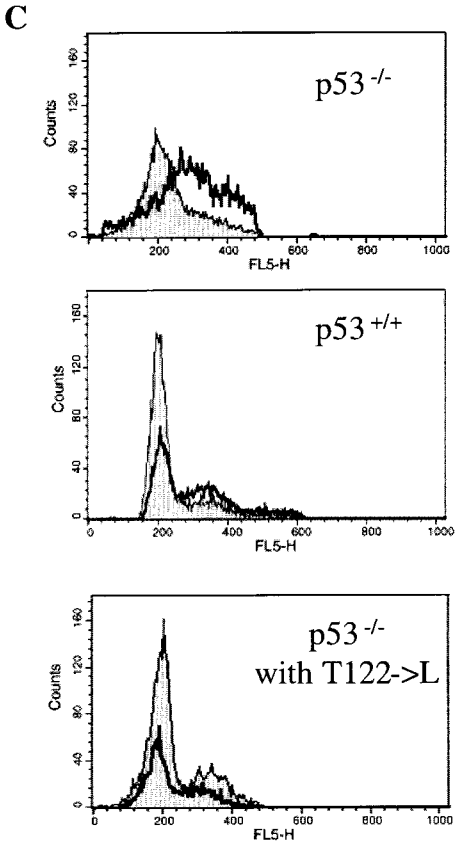


Figure 2 Effects of T122→L on UV-induced apoptosis and cell cycle arrest in MEFs. $p53^{+/+}$ and $p53^{-/-}$ MEF cell lines expressing p53 mutant alleles were mock- or UV-irradiated at the indicated doses. Cells were collected 48 h after treatment and prepared for FACS analysis by 7-AAD and Hoechst double staining. (**a**,**b**) The histograms show the percentage of apoptotic cells in each cell line after the different UV treatments. (**c**) Cell cycle profiles of non-apoptotic cells that were mock-treated (solid area) or UVB-irradiated (25 J/m^2) (black line). Presented on the Y-axis are the numbers of cells counted at each point

T122→L p53 protein has altered transactivation specificity

We evaluated transactivation of a luciferase reporter gene under the control of the human *p21*, *MDM2*, *Bax*, or *c-Fos* promoters, or the *RGC* RE. $p53^{-/-}$ MEFs were subjected to transient transfection by either the wild-type, T122→L or the R270→C alleles, along with a plasmid containing a luciferase reporter downstream from one of the p53-responsive promoters. The luciferase activity was determined in protein extracts 48 h later. The average activity relative to wild-type p53 and the standard deviation of at least three independent experiments are presented in Figure 3a. Although the large variability in this experiment prevents a reliable assessment of subtle differences, this analysis suggests that the T122→L mutant protein exhibits an altered transactivation pattern. As expected, wild-type p53 protein was able to activate transcription from all the promoters, while R270→C was clearly deficient. The T122→L form failed to activate either the *RGC* cassette or the *p21* promoter. Surprisingly, the protein was able to activate both the *MDM2* and the weaker *Bax* promoter. Over-expression of wild-type p53 represses the *c-Fos* basal promoter (described in (Ginsberg *et al*., 1991) and Figure 3a). The T122→L and the R270→C alleles failed to repress the *c-Fos* promoter. The first intron of the *c-Fos* gene also contains a p53 RE and wild-type p53 at near physiological levels of expression can activate a construct that includes the luciferase reporter gene fused to this region (Elkeles *et al*., 1999). The T122→L allele, but not R270→C, activated this RE, though at reduced levels compared to wild-type p53 protein (Figure 3a). Consistent with the transactivation results both wild-type and mutant T122→L p53 proteins prepared from stably infected $p53^{-/-}$ MEFs bound a p53 consensus RE while R270→C protein did not (data not shown).

Transactivation of *Bax*, *p21*, *RGC* and *MDM2* was also examined in Saos-2 cells using a similar approach, except that luciferase activity was measured in protein extracts obtained 24 h after transfection. Levels of p53 protein were high at this time and similar for all three alleles (see Figure 3b). Results were comparable to those observed with MEFs for both the T122→L and the R270→C mutants, except that the T122→L allele also strongly activated *p21* in the Saos-2 cells (data not shown). This difference between MEF and Saos-2 cells for *p21* might reflect cell type-dependent effects. The level of endogenous *p21* induction was measured in Saos-2 cells by Western blot analysis. Wild-type *p53* expression led to strong induction of the *p21* gene while the R270→C mutant failed to induce *p21* (Figure 3b). The T122→L allele exhibited a low level of induction compared to wild-type p53. The difference between ectopic and endogenous gene results may be due to the high copy number of the luciferase reporter and location on a plasmid, which might overestimate the transactivation potential of *p53* alleles that retain partial activity.

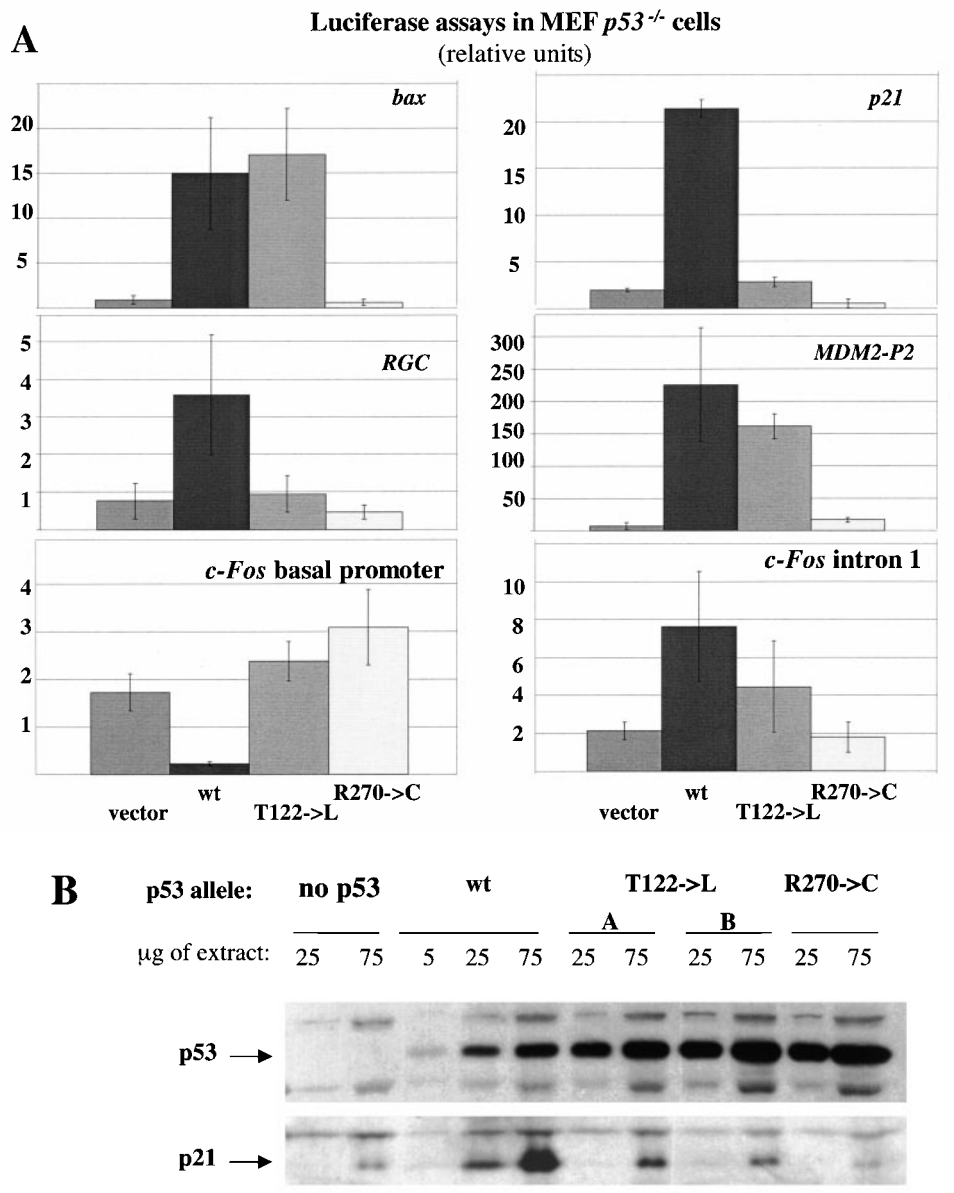


Figure 3 (**a**) T122→L transactivation activity in MEFs. MEF cells were transfected using the FuGENE^{TM} 6-transfection reagent, with 2 μg of *p53* expression vector and 4 μg of reporter plasmid and recovered after 48 h. Relative average luciferase units and standard deviations for at least three independent experiments are presented. The six promoters tested are indicated. T122→L p53 transactivation activity is compared to wild-type p53 and the R270→C mutant. (**b**) p53 expression levels and induction of the *p21* gene in Saos-2 transient transfectants. Protein extracts were prepared 24 h after transfection. p53 and p21 levels (pAb-C19) were determined by Western blot analysis

The T122→L allele exhibits novel transactivation properties in a yeast-expression assay

As shown above, the T122→L allele falls into the category of p53 mutant proteins that retain partial activity (Ludwig *et al*., 1996). However, contrary to previously described mutants that showed partial function (Blagosklonny *et al*., 2001), it appears to retain activity with the weaker *Bax* promoter but not with the stronger *p21*. Furthermore T122→L is the first example of a tumor hotspot mutant that exhibits this type of altered transactivation function as a result of an amino acid change in the L1 loop–S2 strand region, which is part of the highly conserved domain II. Interestingly, similar functional results were reported for the human S121→F p53 mutant (corresponding to S118 in mouse p53), also in the L1 loop (Freeman *et al*., 1994; Kaeser and Iggo, 2002). However, S121→F has never been found in tumors and its ectopic expression led to enhanced apoptotic response compared to wild-type p53 (Saller *et al*., 1999).

These results prompted us to characterize extensively the *in vivo* transactivation capacity of the T122→L

protein with a large variety of REs using a yeast-based reporter system. Previously, three yeast strains were developed that contained a copy of the *ADE2* color (red/white) reporter gene whose transcription was dependent on p53 protein binding to an upstream *RGC*, *Bax* or *p21* RE (Flaman *et al*., 1995, 1998). More recently we developed a system in yeast that provides opportunities to examine functional features of mutants in the DNA binding domain. *p53* expression is controlled by a rheostatable *GAL1* promoter (rather than a strongly expressing promoter) so that relative p53 transactivation ability on various REs can be assessed (Inga *et al*., 2001). This system was adapted to study the effects of the mouse T122→L allele on transactivation and the number of REs examined was extended to 12 using isogenic strains each containing a different *p53* RE (see Table 1).

While transactivation could be examined at reduced levels of expression, higher expression of the T122→L allele from the *GAL1* promoter completely prevented yeast growth, irrespective of the strain background and the affinity for the p53 RE in the *ADE2* reporter gene (Figure 4, Table 1). Under the same conditions expression of wild-type mouse p53 protein resulted in small colonies (not shown), as did human p53 protein (similar to our previous studies (Inga and Resnick, 2001)). In contrast, the non-functional mutant allele R270→C had little effect on growth (data not shown). We also constructed yeast expression vectors with *p53* under the control of the moderately expressed, constitutive *ADH1* promoter. As expected from other reports (Scharer and Iggo, 1992), wild-type p53 protein did not affect yeast growth at this level of expression while the T122→L allele prevented growth (data not shown). This strong growth-inhibiting phenotype is similar to that found for *supertrans*/toxic p53 alleles which we previously described (Inga and Resnick, 2001).

We also evaluated the transactivation potential of the T122→L allele with respect to nine human and three murine p53 REs at low levels of induction from the *GAL1* promoter. Each of these response elements, which belong to the p53 consensus but differ in nucleotide sequence (el-Deiry *et al*., 1992), were derived from the regulatory regions of p53-regulated genes involved in cell cycle arrest, DNA repair and apoptosis induction. While the human REs may not be identical to those in the homologous mouse genes, they provide

Table 1 Transactivation activity of wild-type and T122→L p53 and 12 *p53* REs under conditions of variable p53 induction, using the *GAL1* promoter

p53 RE	*Minimum galactose level (%) required to detect transactivation with:* *wild type p53*	*T122→L*	*T122→L activity relative to wild type p53*[b]
p21-5′	0.004	0.001	↑↑
p21-3′	0.008	(>0.032)[a]	–
PCNA	0.002	0.004	↓
GADD45	0.001	0.0005	↑
Bax	0.008	0.002	↑↑
PIG3	0.064	0.032	↑ ?[c]
IGF-BP3 box A	0.032	0.002	↑↑↑↑
AIP1	0.001	0.004	↓↓
m-FAS	0[d]	0.001[d]	↓
cFOS	0.008	0.008	=
MDM2	0.004	(>0.032)[a]	–
3×RGC	0.004	(>0.032)[a]	–

[a]The ability to detect transactivation at galactose levels >0.032% by the T122→L mutant cannot be determined due to general toxicity of the mutant (see Figure 4). There is no transactivation at 0.032% galactose; [b]Enhanced, equal, reduced or loss of function is represented by ↑, =, ↓, and –, respectively. Each arrow indicates a twofold difference in galactose level needed to detect transactivation; [c]Since wild-type p53 shows transactivation with *PIG3* at high expression levels, the poor growth of cells expressing the T122→L allele makes color analysis less reliable; [d]With expression of wild-type p53, colonies are already white on raffinose plates. With T122→L, colonies become pink at 0.001% galactose, but they remain pink at all galactose concentrations for which there is growth

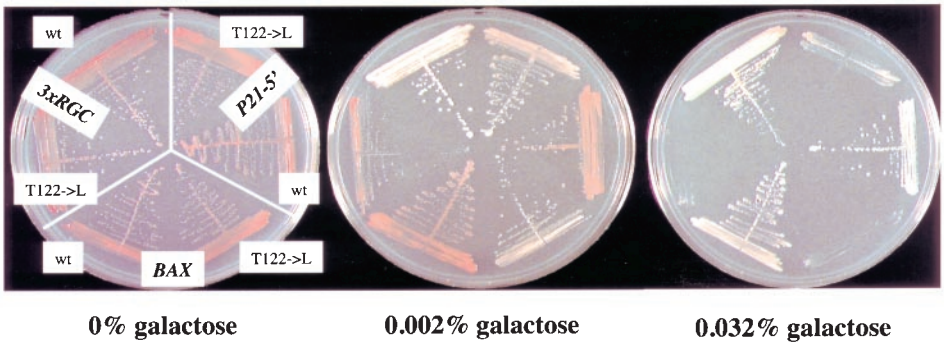


Figure 4 Transactivation potential of wild-type and T122→L mutant p53 proteins under condition of variable expression in the *yIG397*, *yPHp21*, and *yPHbax* strains. *GAL1::p53* expression vectors were transformed into strains containing *3xRGC*, *BAX* and *p21::ADE2* reporters. Transformants were streaked out for single colonies on plates containing 2% raffinose as the carbon source plus the indicated amounts of galactose inducer. A low amount of adenine (5 mg/l) in the plates allows the assessment of p53 dependent transactivation (white/pink vs red colonies). When grown on medium lacking galactose, all colonies are red. On 0.002% galactose T122→L is defective for transactivation with *3xRGC* (red colonies compared to white colonies obtained with wild-type p53 transformants), but it exhibits enhanced activity with both *p21* and *BAX* on the 0.002% galactose plate (pink colonies vs the red colonies with wt *p53*). On 0.032% wt p53 efficiently induces all three promoters, while this level of expression prevents growth of T122→L transformants

the opportunity to investigate differences in binding affinity/transactivation capacity between wild-type and mutant *p53* alleles.

Independent yeast transformants containing the *p53* expression vector were streaked for single colonies on plates containing raffinose (which leads to a higher basal level of *GAL1* expression compared to glucose) plus different amounts of the galactose inducer (e.g., see Figure 4). Growth and levels of transactivation, indicated by red, pink or white colonies, were determined and compared to wild-type *p53* expression (Table 1). Transactivation analysis at low expression levels revealed differences in activity of wild-type p53 with the various REs. For example, transactivation with the *GADD45*, *AIP1*, *p21-5′* REs was observed at lower levels of p53 expression than with the *p21-3′*, *BAX*, and *MDM2* REs. Under conditions of high expression (0.032% galactose) where cells containing T122→L do not grow, wild-type p53 showed detectable transactivation with all REs. However, transactivation of the *PIG3* and *IGF–BP3* REs by wild-type p53 was reduced even at 0.3% galactose (i.e., pink colonies) (data not shown). It is worth noting that human and murine wild-type p53 (that share an overall 89% identity in the DNA binding domain) showed the same relative differences in transactivation capacity with these REs (data not shown).

Multiple differences were noted between the T122→L mutant and wild-type alleles that were revealed only at various low levels of expression. Surprisingly, transactivation was enhanced for the REs *p21-5′*, *GADD45*, *BAX*, *IGF–BP3-boxA* and possibly *PIG3*, while it was reduced but clearly detectable with the *PCNA*, *AIP1* and *m-FAS* REs. Wild-type levels of transactivation were observed with *cFOS*. Finally, there was no transactivation (colonies remained red) with the *p21-3′*, *3xRGC*, and *MDM2* REs, indicating a loss of binding to these elements. The increased transactivation with some p53 REs did not correlate with the relative transactivation capacity of wild-type p53 protein towards the various elements. In fact, transactivation was enhanced with both strong (e.g., *GADD45*, *p21-5′*) and weak (e.g., *BAX*, *IGF–BP3*) p53REs.

Since T122→L showed both reduced and enhanced functional activity it is unlikely that differences in protein stability explain the transactivation results. In a separate study with wild-type p53, we established that the amount of p53 is directly proportional to the amount of inducer, so that differences in the levels of galactose required to detect transactivation can be used to estimate relative transactivation capacity towards different REs (Inga *et al*., in preparation). Based on the amount of p53 required for transactivation, T122→L is ~3 times less active than wild-type p53 for transactivation at the *AIP1* response element and two times less active with *PCNA* and *mFAS*. Interestingly, T122→L showed partial function (pink colonies) with this latter RE at low expression, but transactivation did not improve by increasing the protein amount. The mutant protein was ~5 and three times more active with the *Bax* and p21-5′ REs, respectively. The largest difference, ~10-fold increase, was found with *IGF-BP3*. This is one of the weakest REs with wild-type p53 but among the strongest with T122→L. The sequence of IGF–BP3 RE (5′ AAACAAGCCacAACATGCTT-3′) has two mismatches from the consensus, both at the first position of the p53-monomer binding sites. It is unclear how the T122→L change can improve p53 binding to *IGF–BP3*. Molecular modeling of the equivalent human T122→L change predicted that the mutant protein would be non-functional (Reis *et al*., 2000).

Since most of the functional assays have been performed in isogenic strains that differ only by the small RE sequences, we conclude that the T122→L amino acid change leads to altered DNA binding specificity towards many REs.

Discussion

Novel features of the T122→L mutation revealed in mammalian and yeast cells

To better understand the importance of the unusual hotspot T122→L change in p53, we utilized a variety of approaches to assess the functional consequences of this mutation. These approaches in yeast and mammalian cells can be used to characterize the many functionally altered p53 alleles that are likely to appear in human tumors.

Our results indicate that the hotspot T122→L mutant protein has novel functional features relative to wild-type and other *p53* hotspot mutant proteins. The mutant protein exhibited transactivation in luciferase assays, induced partial cell cycle arrest (but not apoptosis) in response to UV radiation, and was poorly tolerated when ectopically over-expressed in Saos-2 cells. These results in mammalian cells suggest a separation of functions since there was loss of apoptosis induction. This specific change in apoptotic response has also been described for a small number of p53 mutations that when over-expressed in tumor cell lines exhibited normal or partial activity toward the *p21* promoter in reporter assays, but lacked transactivation of the *Bax* promoter (Blagosklonny *et al*., 2001; Ludwig *et al*., 1996). While the induction of *p21* can be sufficient for cell cycle arrest, the p53-dependent apoptotic response appears to require the concerted activation of several genes including *Bax* (Munsch *et al*., 2000). Transactivation by T122→L p53 differs from these rare partial function mutations in that it has no activity towards *p21* in MEFs or has greatly reduced activity in Saos-2 cells towards the *p21*, but shows wild-type activity with the *Bax* promoter in both cell lines. Yet, its ectopic expression only leads to G1 arrest and there is no apoptotic response.

Results obtained with over-expressed *p53* alleles at non-physiological levels of protein must be viewed cautiously, especially in light of the complexity of *p53*

responses and their regulation, and the activation hierarchy of the many target genes (Vogelstein *et al*., 2000; Zhao *et al*., 2000). For example, p53 REs often deviate from the loose consensus sequence in terms of the actual sequence and the number and the arrangement of elements, resulting in variation in transcriptional activation (Szak *et al*., 2001; Thornborrow and Manfredi, 1999; Wieczorek *et al*., 1996). In addition, the level of *p53* expression, the extent of specific post-translational modifications in the pool of nuclear p53 proteins and the availability of cofactors might affect the biological outcome of the *p53* response by establishing further discrimination between target genes (Thornborrow and Manfredi, 2001).

The yeast p53 functional assay with moderate p53 expression under the *ADH1* promoter has also been used to characterize many tumor alleles and identify p53 mutant forms retaining function with specific REs (Campomenosi *et al*., 2001; Flaman *et al*., 1998). While several alleles appeared wild-type with *p21* and mutant with *Bax* RE, the opposite phenotype was never observed. In addition, none of these mutants appeared to affect yeast growth.

The novelty of the T122→L mutant phenotype led us to characterize the transactivation potential of this allele using the rheostatable *p53* expression system developed in yeast (Inga *et al*., 2001). This assay previously revealed several *p53* mutations that are toxic in yeast at the moderate expression levels found with the frequently used constitutive *ADH1* promoter (Inga and Resnick, 2001). At low expression levels, the toxic mutants often exhibited enhanced transactivation and altered promoter specificity. The T122→L allele is similar to these *supertrans*/*toxic* mutants. It is located in the conserved domain II and it prevents growth of yeast, even at moderate levels of expression.

The transactivation analysis at various low levels of expression identified numerous changes of the T122→L protein relative to wild-type p53. These included enhanced or *supertrans* activity for several REs, and reduced or no transactivation for others (Table 1). For example, the human V122→A mutant allele (corresponding to V119 in mouse p53) showed a transactivation pattern similar to T122→L (unpublished results), although there were increased activities with *hFAS* and *mFAS* REs. V122→A was wild-type for growth suppression in Saos-2 cells, and it has never been found in tumors (Inga and Resnick, 2001).

A comparison of transactivation results showed a good correlation for activation of *BAX*, *c-Fos* and *RGC* p53 REs by T122→L in yeast and mammalian cells (Figure 3 and Table 1), while there were differences with *p21* and *MDM2*. However, the two *p21* REs from the human *p21* promoter were examined separately in yeast and opposite activities were found with T122→L (i.e., enhanced and defective transactivation). The contribution of both elements to the activity of the complete promoter is uncertain. The yeast results suggest that efficient activation of the *p21* promoter requires the interaction of p53 with both REs. Interestingly, the p21-3′RE is about 1 Kb closer to the transcription start site and may have an important role in stimulating chromatin modifications near the TATA box (Espinosa and Emerson, 2001). In the case of *MDM2* only one of two REs found in the p53-responsive intronic promoter (Zauberman *et al*., 1995) was tested in yeast. It is possible that T122→L retains activity with the other *MDM2* RE and hence manifests activity with the complete promoter in mammalian cells. Gene expression profiling in mouse skin cells at physiological levels of *p53* expression might be informative in characterizing how the T122→L mutations alters the pattern of expression of *p53*-regulated genes.

Preference for the T122→L p53 mutation in $Xpc^{-/-}$ $p53^{+/-}$ cells

Several factors can influence the appearance of mutation hotspots in genes. These include (i) the likelihood that a nucleotide(s) is damaged; (ii) the ability of one or more DNA repair systems to detect and process the damage; (iii) the miscoding potential of the lesion and (iv) the functional consequences of the mutation (Holmquist and Gao, 1997). Given the well-established role of *p53* in tumor suppression, the functional consequences of mutations in this gene are expected to significantly contribute to selection (Rodin *et al*., 1998). The predominance of *p53* tumor mutations located in the DNA-binding domain and their predicted effect in reducing or preventing DNA binding strongly supports the view that p53 transactivation function is critical to tumor suppression (Ko and Prives, 1996). Several *p53* mutation hotspots have been identified in tumors (Hernandez-Boussard *et al*., 1999), and some have been associated with specific DNA changes such as DNA damage or methylation of CpG sequences (Hussain and Harris, 1999). Some hotspots that are associated with tumorigenesis result in gain of function (Lee *et al*., 2000; Murphy *et al*., 2000).

Mutations in *p53* are frequently observed in non-melanoma skin cancers, particularly in squamous cell carcinomas, in which *p53* mutant clones can be detected in normal and pre-malignant cells (Brash and Ponten, 1998). *p53* mutational hotspots in skin tumors have revealed a distinctive fingerprint that is a hallmark of UV radiation-induced mutagenesis, namely tandem mutations at dipyrimidine sites (Sage *et al*., 1996). It has been proposed that mutation of one *p53* allele in cells exposed to chronic UV radiation confers a phenotypic advantage due to a partial inhibition of the wild-type protein leading to less apoptosis of damaged 'sunburn' cells (Brash *et al*., 1996).

The T122→L hotspot in $Xpc^{-/-}p53^{+/-}$ mice is clearly different from other *p53* mutation hotspots and is likely the result of altered DNA repair and/or selection for the unique function(s) of this allele. Defective nucleotide excision repair (NER) increases the risk of UVB-induced squamous cell carcinomas in mice (Cheo *et al*., 2000). While the T122→L amino acid change is infrequent in $Xpa^{-/-}p53^{+/-}$ mice (D

Nahari and EC Friedberg, unpublished observations), it is the predominant *p53* mutation hotspot in skin cancers induced by UVB irradiation in $Xpc^{-/-}p53^{+/-}$ mice (Reis *et al*., 2000). This difference could result from a specific requirement for XPC protein for the repair of a minor type of UV-induced lesion at a non-dipyrimidine site. Alternatively, or additionally, the selective pressures for p53 inactivation during skin carcinogenesis might differ between the *Xpc*- and *Xpa*-defective mice. $Xpc^{-/-}$ cells are defective only in global genome repair (GGR) while $Xpa^{-/-}$ mutants are also defective in transcription-coupled repair (TCR) (Volker *et al*., 2001).

Stalled RNA polymerase at the site of a lesion, inhibition of transcription and/or prolonged association of p53 proteins with TFIIH might contribute to p53 activation and induction of apoptosis. Differences have been observed in the cellular responses of *Xpa* and *Xpc* mutants to UV radiation. While large amounts of p53 accumulate in the nucleus and enhanced levels of apoptosis are observed in UV-irradiated $Xpa^{-/-}$ keratinocytes, the apoptotic response of $Xpc^{-/-}$ keratinocytes is similar to that observed in wild-type cells (Wijnhoven *et al*., 2000). Moreover, like wild-type cells, UVB-irradiated $Xpc^{-/-}$ but not $Xpa^{-/-}$ keratinocytes can progress through S phase, a stage of the cell cycle where p53 function might be reduced (Gottifredi *et al*., 2001). However, the defect in GGR coincides with subsequent arrest in G_2, likely resulting from persistent DNA damage (van Oosten *et al*., 2000).

Given the potential role of p53 in affecting DNA repair (Offer *et al*., 2001; Smith *et al*., 2000), *p53* mutations that result in less apoptotic activity while retaining DNA repair stimulation and cell cycle control may have a higher likelihood of being oncogenic. Interestingly, the transactivation results with T122→L in yeast (Table 1) showed reduced activity with the RE of both *AIP1* and *mFAS*, two important genes for UV radiation-induced apoptosis in skin cells (Hill *et al*., 1999; Oda *et al*., 2000), and slightly enhanced activity with the RE of *Gadd45* gene, that instead contributes to DNA repair (Smith *et al*., 2000).

The reasons for the genetic background contributing to the appearance of the T122→L hotspot are not obvious. However, it is interesting to speculate on this possible contribution in light of the following observations: (1) the levels of p53 protein produced in wild-type and various DNA repair deficient mouse mutants in response to UV are different; and (2) specific responses such as apoptosis can be enhanced at high levels of p53 (Lane, 2001; Zhao *et al*., 2000). In order to induce skin cancer, mice were chronically UV-irradiated. This is expected to result in p53 stabilization/activation and subsequent downstream p53-regulated responses. Mice with the following six genotypes were examined: wild-type, $p53^{+/-}$, $Xpc^{-/-}$ $p53^{+/+}$, $Xpc^{-/-}$, $p53^{+/-}$, $Xpa^{-/-}$ $p53^{+/+}$ and $Xpa^{-/-}$ $p53^{+/-}$ (Reis *et al*., 2000; Takeuchi *et al*., 1998) (Nahari and Friedberg, unpublished results). Since the amount of unrepaired damage influences the extent of p53 induction, the $Xpa^{-/-}$ cells would have the highest levels of p53 and apoptosis. The p53 levels would be expected to be reduced in $Xpc^{-/-}$ cells and somewhat lower in wild-type cells where repair is most extensive. The appearance of the T122→L p53 mutation in the $Xpc^{-/-}$ $p53^{+/-}$ background might result in greater viability compared to $Xpa^{-/-}$ $p53^{+/-}$ cells because of less p53 protein and reduced ability to induce apoptosis by the mutant protein. The partial capacity by T122→L for cell cycle arrest and DNA replication/repair functions might further reduce p53-independent apoptosis. Since the mutation appears to be recessive, there would be no selective advantage of a T122→L mutation in a $p53^{+/+}$ background. There have been only two cases of tumor-associated T122→L mutations in $Xpc^{-/-}$ $p53^{+/+}$ mice and in both the other allele had an independent inactivating mutation (Reis *et al*., 2000). In $p53^{+/+}$ cells there would be a strong selective pressure for dominant-negative, non-functional p53 mutations (such as R270→C) that may also affect p53-independent (e.g., *p73*-mediated) apoptosis. Interestingly, although the R270→C mutation is frequent in UVB-induced skin tumors from wild-type, and $p53^{+/+}$ $Xpc^{-/-}$ or $Xpa^{-/-}$ mice, it was not found in the $Xpc^{-/-}$ $p53^{+/-}$ background (Reis *et al*., 2000; Takeuchi *et al*., 1998).

Implications of the T122→L mutation

We have determined that the T122→L mutant p53 is altered in its ability to recognize and transactivate a variety of test REs and have shown that its activity in the yeast functional assay is different from any of the previously studied hotspot tumor *p53* alleles. Our results suggest that it is unlikely that the high incidence of T122→L mutations in tumors is solely the result of specific DNA damage, a defect in DNA repair, and/or a reduction in wild-type *p53* gene dosage. The altered DNA binding affinity and specificity of the T122→L mutant protein suggests that it would lead to an altered pattern of expression of downstream *p53*-regulated genes *in vivo* in tumor cells and that this contributes to its selection. These results suggest that other *p53* tumor mutations may also reflect functional changes that are uniquely advantageous in the tissue in which they arise. In this regard, it is interesting that in breast cancers identified in BRCA1 families, novel *p53* mutations have been detected (Crook *et al*., 1998), some of which have subtle transactivation defects (Inga and Resnick unpublished).

We propose that the yeast functional assay with inducible p53 expression is a relevant and sensitive screening tool for evaluating *p53* tumor mutations that retain partial function. Transactivation analysis with variable expression of *p53* using different REs under isogenic conditions provides for broad characterization of the relative transactivation capacity of *p53* alleles. This information may be valuable for structure/function studies, tumor diagnosis, prognosis and clinical intervention. When combined with our recent *delitto perfetto* system for rapid *in vivo*, site-directed

mutagenesis (Storici *et al*., 2001), this assay may prove useful for developing a detailed functional classification of tumor-associated *p53* alleles.

Materials and methods

Plasmids, MEF and Saos-2 cell transfections, growth suppression assays, luciferase assays, and Western blots

The mouse wild-type *p53* cDNA and the T122→L and R270→C mutant cDNAs were cloned into plasmid pCI-Neo (Promega, Madison, WI, USA) under control of the strong constitutive *CMV* promoter. These vectors were used for transient transfections in MEFs and for all experiments in Saos-2 cells. $p53^{-/-}$ and $p53^{+/+}$ MEFs were derived from 12-day-old embryos by culturing homogenized samples in DMEM with 10% fetal calf serum at 37°C under 5% CO_2. For growth suppression assays, MEF cells were infected by the retroviral vector pBabe–PURO expressing mutant *p53* alleles under the LTR promoter. Puromycin-resistant colonies were selected. For the luciferase assays, 8×10^5 cells were plated 24 h before transfection. The cells were then transfected using the FuGENE^{TM} 6 transfection reagent (Roche, Indianapolis, IN, USA), 2 μg of pCI–Neo *p53* expression vector and 4 μg of reporter plasmid and recovered after 48 h. Luciferase activity from 10 μl of cell lysates was assayed with the Luciferase assay system (Promega) using a Rosysenthos Lucy 2 type luminometer.

The osteosarcoma derived *p53* null cell line Saos-2 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in McCoy's 5A medium with 15% FBS serum (Life Technologies, Grand Island, NY, USA). T25 cm^2 cell cultures flasks were seeded and transfected at 60–80% confluence by the lipofectamine reagent (Life Technologies). For stable transfections, 1 μg of purified plasmid DNA was used. Cells were selected by adding G418 (Life Technologies) at 0.5 mg/ml after one day of recovery in complete medium following removal of lipofectamine. Colonies were stained with Coomassie Blue after 2–3 weeks. Independent stable transfectant clones were obtained by limiting dilution in 96-well plates. Luciferase assays were performed using 20 ng of *p53* expression plasmid and 500 ng of reporter vector in 12 well-plate clusters. Plasmids pGL1012, pGL1138 and pGL–NA containing a 370 bp fragment of the human *BAX* promoter, a 2.3 kb region from the *p21* promoter, and the *MDM2–P2* promoter regulating the luciferase gene, respectively, were kindly provided by Dr Moshe Oren. Plasmid PG13 containing the luciferase gene under p53 control through 13 copies of the *RGC* sequence was kindly provided by Dr Bert Vogelstein.

Cells were recovered after 24 h, lysed, and protein concentration was measured with the Bradford assay (Biorad, Hercules, CA, USA) according to the standard protocol. Luciferase activity was measured with the Luciferase Assay System (Promega, Madison, WI, USA) using the single photon monitor program in a scintillation counter (Beckmann, Irvine, CA, USA). After subtraction of the blank reading, arbitrary light units relative to 1 ng of protein extract were determined. The same or similarly obtained protein extracts were used for Western blot analysis. Precast SDS–PAGE gels (PAGE–ONE, Owl Separation Systems, Portsmouth, NH, USA) were used for electrophoresis. Proteins were transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) using a semi-dry electroblotter (Owl Separation Systems) according to instructions. p53 was detected using both pAb1801 and DO-1 monoclonal antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) at a 1 : 2000 dilution. p21 was detected using C-19 polyclonal antibody at a 1 : 1000 dilution (Santa Cruz). Immunodetection was performed using the ECL kit (Amersham, Cleveland, OH, USA) according to protocol.

Apoptosis and cell cycle assays in MEFs

$p53^{-/-}$ MEF cells were stably infected using the retroviral vector pBabe–PURO that was empty or expressed either the T122→L or the R270→C mutant proteins under the LTR promoter. 1×10^6 cells per 15 cm^2 dish were plated and were irradiated or mock-treated 24 h later. The cells were harvested 48 h after treatment. Determination of death and DNA content was performed by double staining the cells with 7-AAD (Molecular Probes) and Hoechst (Molecular Probes). Levels of fluorescence staining were assessed by flow cytometry (FACScan Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Yeast strains, plasmids, media and reagents

The haploid *S. cerevisiae* strain *yIG397* (*Mat**a** ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3-1 URA3 3xRGC::CYC1::ADE2*) (Flaman *et al*., 1995) contains an integrated copy of *ADE2* under control of a minimal *CYC1* promoter. Three copies of the human p53 response DNA element found at the ribosomal gene cluster are inserted in the upstream region of the promoter. Thus, *ADE2* is under p53 transcriptional control so that transactivation by p53 mutant proteins can be easily scored based on the color of yeast transformants on suitable plates. Colonies expressing wild-type p53 grow as adenine prototrophs yielding white colonies on plates containing a limiting amount of adenine while small red colonies appear when a nonfunctional p53 is expressed. The haploid strains, *yPH-p21* (*Mat**a** ura3-52 lys2-801 ade2-101 trp1–Δ63 his3–Δ200 leu2–Δ1, URA3::p21-RE::pCYC1::ADE2*) and *yPH-bax* (*Mat**a** ura 3-52 lys2-801 ade2-101 trp1–Δ63 his3–Δ200 leu2–Δ1 URA3::bax-RE::pCYC1::ADE2*) (Flaman *et al*., 1998) allow the evaluation of p53 transactivation function as in *yIG397*, but with the *p21* and *BAX* p53 REs controlling *ADE2* transcription, respectively. In order to assess the transcriptional activation of the same *ADE2* reporter gene at a fixed chromosomal locus as a function of specific p53 REs, the haploid strain *yIG397* was modified as follows. A *ura3* mutant that had popped out the *ADE2* reporter gene at the *URA3* locus was obtained. The *ade2-1* gene was deleted by a PCR-based method and a promoterless wild-type copy of the *ADE2* open-reading frame was integrated replacing the *ade2-1* locus. Isogenic derivatives of this strain, identified as *yAFM-RE*, each containing a minimal *CYC1* promoter that replaces the promoter of the *ADE2* open-reading frame fused to a different p53 RE, were constructed. Expression of wild-type p53 in the *yAFM* strains results in the growth of white colonies on plates containing a limiting amount of adenine, since p53 stimulates *ADE2* transcription, while small red colonies appear when nonfunctional p53 is expressed. All the p53 response elements tested in this study are listed in Table 1. The sequence of each response element and a detailed description of the strain construction will be described elsewhere (Inga *et al*., in preparation).

Plasmids pTGmp and pTAmp are yeast centromeric expression vectors for the mouse wild-type p53 under the control of the *GAL1,10* and *ADH1* promoters, respectively. They were constructed from plasmid pLS89 (Flaman *et al*.,

1995) by replacing the human *p53* cDNA with the mouse homologue to obtain pTGmp and then replacing the *GAL1* promoter with the *ADH1* promoter to obtain pTAmp. The T122→L and R270→C mutations were cloned in pTGmp and pTAmp from the pCI-Neo vectors.

Yeast strains were cultured in 1% yeast extract, 2% peptone, 2% dextrose (YPD) or in YPD medium containing 200 mg/l adenine (YPDA) or on selective medium. p53 transactivation was generally determined in synthetic medium containing 5 mg/l adenine. Synthetic medium containing 2% raffinose (Sigma, St. Louis, MO, USA) as carbon source and variable concentrations of galactose was used to test transactivation and growth inhibition by various levels of p53.

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